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PRINCIPAL INVESTIGATOR: Paula H. Stern, Ph.D.

CONTRACTING ORGANIZATION: Northwestern University  
Evanston, Illinois 60208-1110

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| <b>13. ABSTRACT (Maximum 200)</b><br>The goal of the research is to determine the cause of the osteoporosis resulting from excessive thyroid hormone. The question is complex, due to the fact that thyroid hormone is also critical for bone growth. Based upon our previous findings that thyroid hormone increased the production of both the osteoclastogenic cytokine interleukin-6 and the important bone growth factor, insulin-like growth factor (IGF-I), we proposed to determine the roles of these local factors in the effects of thyroid hormone to stimulate both bone growth and bone loss. Also we proposed to determine the pathways by which thyroid hormone increases these local factors in bone. During the second year of the grant we have completed studies that demonstrate that IGF-I mediates anabolic effects of T3 on proline incorporation, alkaline phosphatase and osteocalcin. The decline in IGF-I production that we previously observed at pathophysiologic T3 concentrations could thus represent part of the mechanism for the bone loss. We are continuing studies on the role of IL-6 with the establishment of a model for testing effects on differentiation. Several new strategies for examining T3 effects on IGF-I and IL-6 gene expression, including the molecular reconstruction of the pathway in non-osteoblastic cells are being pursued. |   |  |   |  |
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Paula H. Stern 10/21/98  
PI - Signature Date

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## INTRODUCTION

The subject of the studies is the mechanism of the effects of thyroid hormone (T3) on bone. The purpose of the studies is to determine the roles and pathways of local growth factor and cytokine production in the effects of thyroid hormone (T3) on the formation and breakdown of bone, with the ultimate goal being to elucidate how excess thyroid hormone leads to osteoporosis. The scope of the studies for year 2 was 1) to continue studies on the pathways of T3 induced synthesis of insulin-like growth factor-I (IGF-I) and interleukin-6 (IL-6) (Specific Aims IA and IIA), including promoter analysis and 2) to continue studies on the effects of interference with IGF-I and IL-6 actions on the anabolic and catabolic effects, respectively, of T3. Studies from our and other laboratories formed the immediate background for the proposal. Specifically, T3 has been found to have a dose-dependent biphasic effect on the production of the important bone growth factor IGF-I by bone and osteoblastic cells, the T3 stimulating IGF-I production at low concentrations and producing progressively lesser effects as the T3 concentrations are increased into the pathophysiologic range. Thus, it was conceivable that some of the mechanism of the bone loss resulting from high T3 could be due to the decline in the stimulation of IGF-I production, leading to the loss of the growth factor stimulatory effect. A second postulated mechanism for the bone loss was the increased production of the bone resorbing cytokine IL-6, possibly through an enhancement of its production by interleukin-1.

It is well established that thyroid hormone is a critical regulator of skeletal development and maturation. Evidence demonstrating effects of T3 to promote both the formation and breakdown of bone can be found in clinical observations as well as in vivo investigations in animals (Deftos 1978, Stern 1996). Long-standing juvenile hypothyroidism can cause severe growth retardation when thyroid hormones are not replaced (Mosekilde et al. 1990). T4 treatment in children with congenital hypothyroidism resulted in a positive correlation between bone age and the dose of T4 administered (Heyerdahl et al. 1994). Levels of growth hormone and IGF-I are reduced in hypothyroidism, but treatment with GH alone failed to restore normal growth in hypothyroid children (Gaspard et al. 1978). Although in children and in young animals, excess T3 causes enhanced bone growth (Glasscock and Nicoll 1981, Schlesinger and Fisher 1951), it leads to bone loss in adults (Fraser et al. 1971, Franklyn and Sheppard 1992). It is well established that both bone formation and resorption are markedly increased in hyperthyroidism (Stern 1996). Greater increases in the resorption markers than the formation markers suggest an imbalance between resorption and formation, leading to a net loss of cortical and trabecular bone volume (Garnero et al. 1994). Despite the clinical importance of the effects of T3 on bone, the processes and molecular mechanisms by which this occurs are still poorly understood. T3 stimulates bone resorption *in vivo* and *in vitro*. Excretion of pyridinium cross-links is elevated in hyperthyroidism and in patients on thyroid replacement therapy (Ernst and Froesch 1987). Histomorphometric analyses show increased osteoclast numbers and increases in resorbing surfaces, with loss of trabecular bone volume (Mosekilde and Melsen 1978). T3 stimulates cell replication in both rodent and human osteoblastic cells (Ernst and Froesch 1987, Kassem et al. 1993). At high, supraphysiological concentrations T3 inhibits replication (Sato et al. 1987, Kasono et al. 1988, LeBron et al. 1989).

The osteoblast appears to be the critical target cell for T3 action in bone since T3 fails to activate isolated osteoclasts to resorb in the absence of osteoblasts (Allain et al. 1992). Receptors for T3 were demonstrated by binding studies in rodent and murine osteoblast cell lines and normal

osteoblasts (Sato et al. 1987, Kasono et al. 1988, LeBron et al. 1989, Egrise et al. 1990, Rizzoli et al. 1986, Krieger et al. 1988). The osteoblastic cell lines UMR-106 and ROS17/2.8 express both T3R $\alpha$ 1 and T3R $\beta$ 1 as well as RXR and RAR isoforms (Williams et al. 1994). Transient gene expression assays and treatment of cells with T3, calcitriol and 9-cis-retinoic acid provide evidence that receptor complexes are functional, and even enhancing, in these cells. In recent years it has become apparent that osteoblasts are sites for the synthesis of local growth factors and cytokines, and these substances may mediate the effects of systemic hormones. Two local factors that are particularly likely to be involved in the actions of T3 on bone are insulin-like growth factor-I (IGF-I) and interleukin-6 (IL-6). IGF-I has significant anabolic effects on bone, increasing cell replication and both collagen and non-collagen protein synthesis (Canalis 1980, Hock et al. 1986, McCarthy et al. 1989, Centrella, et al. 1990, Pirskanen et al. 1993). Schmid, et al. reported stimulation of IGF-I by T3 in normal rat calvarial osteoblasts (Schmid et al. 1992). Our own studies demonstrate a dose-dependent, biphasic stimulation of IGF-I secretion in UMR-106 cells and fetal rat bone organ cultures (Lakatos et al. 1993). Klaushofer and colleagues (Klaushofer et al. 1995) found an increase in IGF-I mRNA in MC3T3-E1 cells treated with T3. IL-6, a potent multifunctional cytokine, is a potent stimulator of osteoclast differentiation from precursor cells in human marrow (Kurihara et al. 1990). Hypercalcemia is elicited in nude mice injected with by Chinese hamster ovary cells that had been transfected with the murine interleukin-6 gene (Black et al. 1991). IL-6 is present in high concentrations in patients with Paget's disease of bone (Roodman et al. 1992). Resorption of dentin *in vitro* by giant cells from human giant cell tumors of bone was inhibited by antisense oligonucleotides to IL-6 (Reddy et al. 1994), and both IL-6 and IL-6R mRNA are higher in Pagetic osteoblasts and osteoclasts than those from normal remodeling bone (Hoyland et al. 1994). Other pathologic states in which IL-6 appears to have a role in bone resorption include multiple myeloma, rheumatoid arthritis and Gorham-Stout disease (Manolagas and Jilka 1995). We have found that T3 potentiates the IL-1 $\beta$ -stimulated production IL-6 in bone (Tarjan and Stern 1995). In collaborative studies with Dr. Peter Lakatos at Semmelweis University Medical School in Budapest, Hungary, we have also found that circulating IL-6 is higher in hyperthyroid (Graves disease and toxic nodular goiter) than in euthyroid premenopausal women (Lakatos et al. 1997). Monocytes from the patients with Graves disease or toxic nodular goiter had elevated IL-6 production compared with controls (Lakatos et al. 1997). Other studies have shown the potentiation by T3 of IL-1-stimulated IL-6 production in human osteosarcoma cells (MG-63) and also noted increases in IL-6 and IL-6 soluble receptor in hyperthyroid patients (Passeri et al. 1995).

Thus, the well-established importance of T3 in the normal development of the skeleton as well as the potential increased fracture risk with thyroid excess make it essential that we understand how T3 acts on bone. At the current time there is virtually no molecular information on how T3 influences bone physiology. The studies we have undertaken are designed to elucidate the molecular mechanisms by which T3 affects the production of IGF-I and IL-6 in bone and to address the critical question of whether T3-stimulated increases in these factors is essential for the actions of T3 in bone.



The specific aims of the project were as follows:

- IA: Determine the mechanism of the effect of T3 to increase IGF-I in osteoblastic cells
- IB: Determine the biological significance of the increased IGF-I for the anabolic effects of T3
- IIA: Determine the mechanism and modulation of T3 potentiation of IL-6 production
- IIB: Determine the biological significance of the T3 potentiation of IL-6 production

The studies have been carried out as a coordinated effort between the laboratories of Drs. Stern and Madison. For the purpose of the report, we have separated the presentation of the results and discussion of the work carried out in Dr. Madison's laboratory (Specific Aims IA and IIA) and those carried out in Dr. Stern's laboratory (Specific Aims IB and IIB).

## **BODY OF THE REPORT**

### **Experimental Methods**

Cell culture: Primary osteoblast cultures were prepared from calvarial bones obtained from 6-7 day old neonatal mice (Sanders and Stern 1996). Mice were housed and sacrificed in accordance with policy at the Northwestern University Animal Care and Use Facility. After the calvaria bones were dissected, cells were released from the bone by 6 sequential 20 min. collagenase digestions. A pool from digestions number 2-6 was collected and seeded into 75-cm<sup>2</sup> flasks for culture in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 5 % fetal bovine serum (FBS) and 100 ug/ml penicillin and streptomycin. Primary osteoblasts from culture passages 1-3 were seeded onto appropriate dishes for experiments. Mouse osteoblastic MC3T3-E1 cells, a mouse immortalized cell line, which has been used by other investigators for studies of thyroid hormone action (Varga et al. 1994, Klaushofer et al 1995). were cultured in  $\alpha$ MEM supplemented with 5% FBS and 50 ug/ml gentamycin. Cells were passaged every 7 days by harvesting with 0.1 % trypsin-EDTA and reseeding in 75-cm<sup>2</sup> flasks. For experiments, MC3T3-E1 cells between passage numbers 3 and 14 were seeded into 60-mm dishes or 24- or 96-well cluster dishes.

For cell differentiation experiments, co-cultures of mouse bone marrow cells and normal neonatal mouse calvarial cells were used. Osteoblastic cells from 1-2 day mice, passaged twice, were mixed at a concentration of 50,000 cells/ml with bone marrow cells from 6-9 week mouse tibiae at 250,000 cells per ml. The mixed cell populations were cultured in a 96 well plate, 0.2 ml/well for 3 days, after which time the medium was removed and calcitriol, 10 nM was added. After an additional 3 day coculture, the medium was removed and the cells washed with 200 ul PBS and fixed with 10% formaldehyde in PBS, for 10 min. The fixative was removed and the cells refixed with ethanol/acetone 1:1 for 1 min. Dishes were dried and the cells stained for tartrate-resistant acid phosphatase using naphthol AS-MX phosphate as a substrate (Udagawa 1989). Cells with more than three nuclei were counted as osteoclasts.

For studies of regulation of IL-6 and IGF-I promoters, neutral, non-osteoblast cells lines (TSA-201, JEG-3 and COS-1) were used..

Culture Media and Hormone Treatments: Cells were cultured in the recommended media in the presence of 5% fetal calf serum (FCS). For experiments involving hormonal manipulation, cells were pre-cultured in hormone depleted media for 12-18 hours, prior to IL-1 and/or T3 treatment.

Two methods were assessed for hormone depletion; charcoal stripping, which removes to near completion all steroid and peptide hormones, and Dowex AF-1-X-10 resin stripping (Samuels et al 1979), which removes T3 and T4 hormones with reasonable selectivity. Charcoal stripping leaves a profoundly nutritionally deprived media and essentially arrests cell division. Resin-treated serum is better at supporting cell growth, but the relative concentrations of other relevant hormones (retinoids, Vitamin D, interleukins) is unknown. In general, resin-treated serum gave the best results, allowing better cell growth and greater IGF-I and Il-6 production and a larger T3 effect.

Time course: Incubations were maintained from 1-5 days, depending upon the experiment. Cell were used at confluency, except for studies on cell replication. MC3T3-E1 cells were maintained in culture with  $\alpha$ -MEM + 5% fetal bovine serum, 50 mg/ml ascorbic acid and 50 mg/ml gentamicin for 18-21 days, to allow differentiation to the osteoblast phenotype before they were used.

Thymidine incorporation: Cells are plated in DMEM or  $\alpha$ -MEM + 5% heat-inactivated fetal bovine serum (FBS) T3/T4-free serum at concentrations that would permit achievement of confluency in 96 hours (20,000 cells/well in a multiwell dish). After 48 hr (at which the cells are at 70% confluency) the medium is changed to 0.1% bovine serum albumin, for an overnight incubation to quiesce the cells. They are then treated with T3 and harvested at 24 hr. Two hours before the end of the incubation, 0.5  $\mu$ Ci/ml  $^3$ H- thymidine is added. Incubations are in 5% CO<sub>2</sub> at 37°C. The cells are then washed 2x with 500 ml cold phosphate-buffered saline (PBS) and reaction is stopped with cold 10% TCA. The TCA is removed and the cells harvested with 750 ml 0.5 N NaOH and 0.1% sodium dodecyl sulfate (SDS). An 100 ml aliquot is used for liquid scintillation counting.

Proline incorporation: Collagen synthesis is estimated by incorporation of  $^3$ H-proline into trichloroacetic acid precipitable protein. Cells are plated in 24-well cluster dishes at 35,000 cells/well. After the culture reached confluence, cells were hormone-depleted by culturing in T3-free medium for 48 hr prior to treatment. Tissues are incubated for 72 hr with the treatments. For the final 2 hr, 1  $\mu$ Ci/ml  $^3$ H-proline is added. The reaction is stopped with 10% TCA, the cells washed, and the incorporation into the TCA-precipitable fraction measured by liquid scintillation counting.

Alkaline phosphatase: Cells are used at confluency in 96 well plates, plated at 16,000 cells/well, except for MC3T3, which are maintained in culture ( $\alpha$ -MEM + 5% resin-stripped FBS, 50 mg/ml ascorbic acid, 50 mg/ml gentamicin for 21 -days). Alkaline phosphatase in cell lysates or culture media was measured by the production of p-nitrophenol from p-nitrophenyl phosphate (Schlossman, et al. 1982). Cells incubated with the selected treatments for 72 hr. Cells are washed with cold PBS. The medium is aspirated, 100 ml diethanolamine, 50 mM, pH 10.5 is added, and ml of 2.5 mM p-nitrophenylphosphate in glycine buffer is added. Medium or tissue extracts are incubated for 30 min with the substrate at pH 10.5 (100 mM glycine/2mM MgCl<sub>2</sub> buffer) and the reaction stopped by the addition of 0.1 N NaOH. Absorbance is read on a Varian spectrophotometer at 410 nm and activity calculated in reference to a standard curve of p-nitrophenol.



Osteocalcin: Osteocalcin secreted by the tissues after 72 hr from the same cultures used for alkaline phosphatase is measured by radioimmunoassay according to procedures provided by Biomedical Technologies. A radioimmunoassay using a goat antiserum to mouse osteocalcin and mouse [ $^{125}$ I]- osteocalcin tracer (Biomedical Technologies, Stoughton, MA) was used. This assay recognizes total osteocalcin.

Statistics: Except where otherwise indicated, experiments were done in triplicate or higher. Data were analyzed by Analysis of Variance with the Newman-Keuls post-test. For all figures, \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$  vs. control; # =  $p < 0.05$ , ## =  $p < 0.01$ , ### =  $p < 0.001$  vs. treatment (IGF or T3).

Western Immunoblotting: IGF-I receptor expression was determined in whole cell lysates of MC3T3-E1 cells by methods described by us previously (Sanders and Stern 1996). The IGF-I receptor antibody was from Santa Cruz.

RT-PCR Assays: RT-PCR measurements were performed as non-competitive reactions which compare the relative expression of an experimental gene to an appropriate control, housekeeping gene (cyclophilin, GAPDH, 18S rRNA). All RT-PCR measurements are performed in the linear range of amplification and the assay is performed in such a manner that the linearity is confirmed before the data is considered valid. Equal aliquots of total RNA are converted to cDNA with reverse transcriptase. Aliquots of the cDNA are subjected to PCR using Taq polymerase, dNTP,  $Mg^{++}$ , and 20 pmole of oligonucleotide primer pairs. PCR products are detected after electrophoresis by phosphorimager quantification of  $^{32}P$ -dNTP incorporated into the product during PCR. To assure a linear assay a set of serial dilutions ( $n=3-5$ ) of the cDNA template (100-500 ng total) is made for each individual sample, spanning 3-4 logs of concentrations. The yield of product from the experimental gene PCR is compared to that of a housekeeping gene for each of the cDNA dilutions (usually cyclophilin). If PCR is occurring in a linear fashion, the ratios of these comparisons remains constant across a dilution series of the sample. The ratios obtained from linear regions of the dilution series are used to determine a Mean and Std. Error for the relative relationship between the experimental gene and the control gene. Comparison can then be made between these relative ratios in different experimental situations.

Transfection, Transient Gene Expression and Luciferase Assays: For transfections studies, cells were plated in 24 well plates, transferred to resin-stripped serum containing media for 18-24 hours. For the IGF-I receptor antisense transfections, cells were transfected using Lipofectamine-PLUS reagent following the manufacturer's (Gibco) protocol. The cells were transfected in serum-free medium for 4 hours and the medium was then replaced with normal growth medium. Levels of IGF-I receptor in transfected cells were assessed 48-72 hours post-transfection. For studies of the IL-6 and IGF-I promoters, a soybean lecithin liposome transfection technique was used to transfect the cells with IL-6-Luc gene constructs/ Luciferase activity in total cellular extracts of cells transfected for 24 to 48 hours was measured for 10 seconds in a luminometer by standard methods.

**Materials:** T3 was from Sigma, IGF-I from Gibco or Biosource, Interleukin-1 from Biosource,  $\alpha$ IR3, a monoclonal antibody specific against the IGF-I receptor was from Calbiochem, JB1, a peptide analog of IGF-I was from Peninsula Laboratories. Antisense oligonucleotides to the rat/mouse IGF receptor were synthesized by the Biotechnology Center at Northwestern University.

### **Assumptions**

The models, materials and design used were based on several assumptions, with some testing of these incorporated into the design:

- 1) It was assumed that the MC3T3-E1 cells were representative of the osteoblast phenotype. This assumption was supported by our similar findings from primary mouse osteoblasts and the MC3T3-E1 cells, and the published literature.
- 2) It was assumed that the parameters we measured would reflect anabolic or catabolic responses. In the case of the anabolic responses, we measured several different parameters. In the case of the catabolic parameters, we used either cultured fetal bones which we assume represents a complete remodeling system, or a co-culture system that is assumed and accepted to be a model for osteoblast differentiation.

### **Procedures**

Covered under Experimental Methods

### **Results and Discussion: Specific Aims IA and IIA (Dr. Madison)**

Our research progress with experiments designed to elucidate the molecular mechanism of T3 action on IL-6 and IGF-I gene expression has been disappointingly limited during the second year of the project. Our initial examination of T3 effects on IL-6 and IGF-I gene expression in a range of osteosarcoma cells was described in the progress report last year. In summary, that work showed that: 1) the magnitude of IL-1 and T3 synergy in controlling IL-6 secretion was limited to a 2-3 fold effect, 2) little or no effect was seen in IGF-1 mRNA production, 3) the IL-6 response was not easily reproducible, being influenced by undetermined factors of cell culture. These results presumably reflect an altered state of differentiation in the various osteosarcoma cell lines, as opposed to the much larger (30-fold) effect seen in primary bone cell cultures. Because of this limited biological response we sought in our second year to more carefully define the cell model and culture conditions and to use two strategies for a more detailed examination of T3 effects on osteoblast-like and osteosarcoma cells. The two strategies are to: 1) use a well defined osteoblast in vitro differentiation model, MC3-T3 cells, to more carefully control the cell and culture conditions, which bedeviled our osteosarcoma experiments. 2) use neutral, non-osteoblast, cell lines (TSA-201, JEG-3, and COS-1) cells to "reconstruct" the molecular regulation of the IL-6 and IGF-1 promoters, transfecting thyroid receptors and other transcription factors in conjunction with a second-messenger strategies to stimulate the IL-6 promoter instead of IL-1.

The first group of experiments uses MC3-T3 cells, a mouse osteoblast cell line capable of undergoing a predictable in vitro differentiation into osteoblast like cells. This model cell line allows for an examination of T3 effects at different stages of differentiation. Early (5 days), mid (10 days), and prolonged (21 days) cultures of cells have been examined for the ability of T3 to augment IL-1 induced IL-6 secretion. The experiments are still in progress, but preliminary analysis suggests that the T3 augmented IL-1 response is significantly augmented in early cultures of the cells. This may in part explain the poor response in the osteosarcoma cells. In most experiments we have measured endogenous IL-6 expression by semi-quantitative RT-PCR. We have also begun to analyze transfected IL-6 - Luc gene constructs, using a soybean lecithin liposome transfection technique. Experiments with metabolic inhibitors and with the IL-6 promoter constructs are in progress to determine the site(s) and promoter location of the T3 effect. We have not yet extended these studies to IGF-1 at a molecular level, however, the results of the IGF-I blocking experiments using the same MC3-T3 cells performed in Dr. Stern's lab provide a discrete framework for an examination of the general features of the T3 effect on IGF-1 production. In addition, Dr. Lowe, our IGF-I collaborator, has constructed a new set of IGF-1 promoter constructs, with significantly higher levels of expression, which are promising for transient gene expression analysis.

The second group of experiments, using "neutral" non-osteoblast cells to perform a molecular "reconstruction" of IL-6 and eventually IGF-I expression are also promising. We have also chosen to examine the thyroid receptor (TR) action in conjunction with the action of the estrogen receptor (ER), because of new observations concerning the role of the ER in control of both of our test genes. Estradiol is a potent inhibitor of IL-1 induced IL-6 gene expression while it is generally thought to promote the expression of IGF-1. A hypothesis has been formed that proposes that the TR action on IL-6 and IGF-I gene activation occurs through modulation of the action of the ER on the respective promoters. The ER action on the promoter has been proposed to occur through interference with NF-IL6 and Nf-kb action on the promoter. The mechanism of ER action on IGF-I has not been defined. The rationale for this approach is the recent discovery of high levels of expression of a unique ER isoform, ER $\beta$ , in osteoblasts, as well as the common clinical observation that T3 effects on bone are more pronounced in the hypogonadal state for IL-6 and in the eugonadal state for IGF-1. We have at our disposal a repertoire of TR and ER $\alpha$  and ER $\beta$  mutant receptors, including dominant negative, constitutively active, and dimerization deficient receptors, which may be useful in defining interactions between the ER and the TR on these respective promoters. Using TSA-201 cells we can obtain high levels of expression of IL-6 and IGF-1 transgenes, but have had difficulty obtaining consistent stimulated expression of IL-6. Experiments with COS and JEG-3 cells are underway.

In summary, our stated goal of defining the mechanism by which thyroid hormone influences IL-6 and IGF-1 gene expression has been severely hampered by the lack of the same magnitude of physiologic response in osteosarcoma cells as has been observed in primary bone cultures. We have devised two strategies to deal with this issue and preliminary results with these approaches are promising.

### **Results and Discussion: Specific Aims IB and IIB (Dr. Stern)**

**Specific Aim IB**, determining the role of IGF-I in the anabolic effects of T3 has essentially been completed, and a manuscript has been submitted and the results presented at one meeting and soon to be presented at a second meeting. Basically, we have shown by three independent

approaches, antibody to the IGF-I receptor, the JB1 peptide antagonist to the IGF-I receptor and antisense oligonucleotides, that interference with IGF-I action in osteoblastic cells markedly attenuates the anabolic effects of T3 on osteoblastic cells. These findings support our hypothesis that IGF-I plays an essential role in the anabolic effects of T3. Interestingly, we found that the phenotypic effects of T3 on alkaline phosphatase, osteocalcin and proline incorporation were not mimicked by IGF-I under the same conditions where they were produced by T3, indicating that IGF-I is a necessary, but not sufficient factor for these phenotypic anabolic effects. The proliferative effects were produced by both T3 and IGF-I and this mitogenic effect of both of these agents was blocked by antagonists to the IGF-I receptor. The results and discussion of Specific Aim IB are from the manuscript: Huang BK, Golden LA, Tarjan G, Madison LD, Stern PH "Insulin-Like Growth Factor I Mediates Anabolic Effects of Thyroid Hormone in Osteoblasts".

#### Results (from manuscript - Specific Aim IB)

Effects of IGF-I receptor antagonists on T3-induced mitogenic activity - 10 nM T3 increased 3H-thymidine incorporation in quiescent MC3T3-E1 osteoblasts. As shown in Figure 1a, a significant effect (17 % increase compared to control) was seen with 4 hour exposure to T3 and this effect was maintained through 12 hour treatment (19 % increase compared to control). 10 nM IGF-I also stimulated proliferative activity in MC3T3-E1 cells (Figure 1b). Quiescent osteoblasts treated with 10 nM IGF-I for 4 and 12 hours showed 39% and 23% increases in 3H-thymidine incorporation respectively.

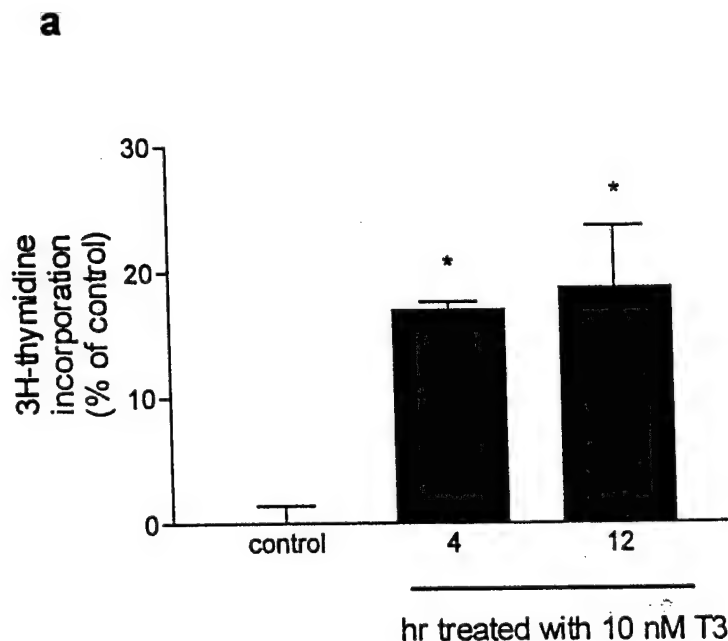


Figure 1a: T3 and IGF-I increase the mitogenic responses of MC3T3-E1 cells. Quiescent MC3T3-E1 cells were treated with 10 nM T3 for 4 and 12 hours in serum-free medium. Control value:  $9731 \pm 243$  dpm/well; \* significantly different from control,  $p < 0.05$ . 3H-thymidine is expressed as percent control. Data represent mean  $\pm$  sem,  $n = 3$  or 4 for each treatment group.

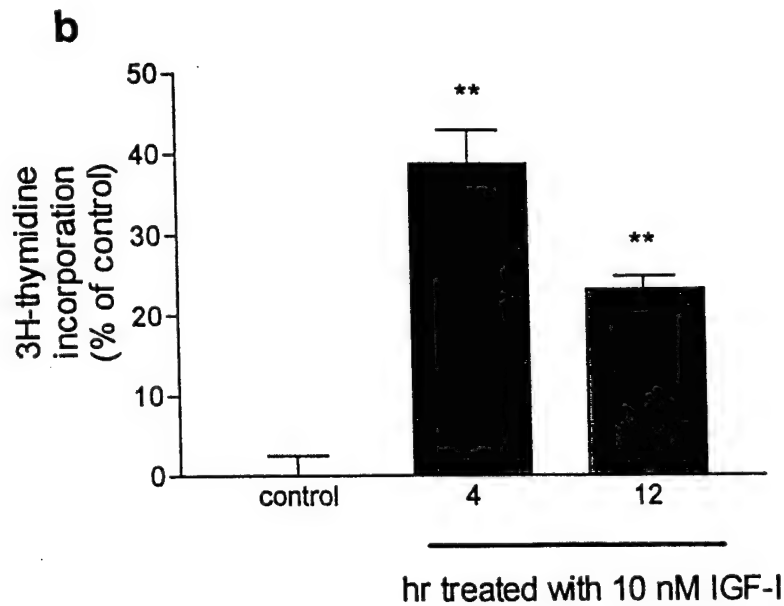


Figure 1b: T3 and IGF-I increase the mitogenic responses of MC3T3-E1 cells. Quiescent MC3T3-E1 cells were treated with 10 nM IGF-I for 4 and 12 hours in serum-free medium. Control value:  $9852 \pm 397$  dpm/well; \*\* significantly different from control,  $p < 0.01$ ; <sup>3</sup>H-thymidine is expressed as percent control. Data represent mean  $\pm$  sem,  $n = 3$  or 4 for each treatment group.

When the cells were co-incubated with 10 nM T3 and an IGF-I receptor antagonist, the stimulatory effect of T3 on <sup>3</sup>H-thymidine incorporation was blocked (Figure 2). Treatment with 10 nM T3 in the presence of 1.5  $\mu$ g/ml of  $\alpha$ IR3, a neutralizing antibody specific for IGF-I receptor (Rohlik et al 1987), attenuated the stimulatory effect of T3 to the level of non-stimulated control cells (Figure 2a).

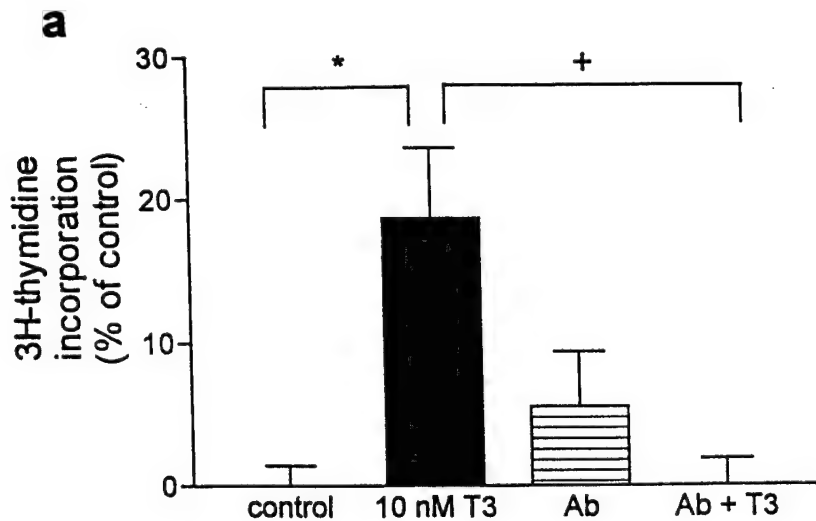


Figure 2a.  $\alpha$ IR3 (Ab) blocks the mitogenic effects of T3 and IGF-I. Quiescent MC3T3-E1 cells were incubated in 10 nM T3 or 1.5  $\mu$ g/ml  $\alpha$ IR3 alone for 12 hours or in the presence of both  $\alpha$ IR3 and T3. Control value:  $9731 \pm 243$  dpm/well; \* significantly different from control,  $p < 0.05$ . + significantly different from 10 nM T3,  $p < 0.05$ . <sup>3</sup>H-thymidine incorporation is expressed as percent control. Data represent mean  $\pm$  sem,  $n = 3$  or 4 for each treatment group.

A similar inhibition was also observed in cells co-treated with IGF-I and  $\alpha$ IR3. The same concentration (1.5  $\mu$ g/ml) of  $\alpha$ IR3 blocked the increased 3H-thymidine incorporation stimulated by 10 nM IGF-I (Figure 2b). Treatment with  $\alpha$ IR3 alone had no effect on basal proliferative responses.

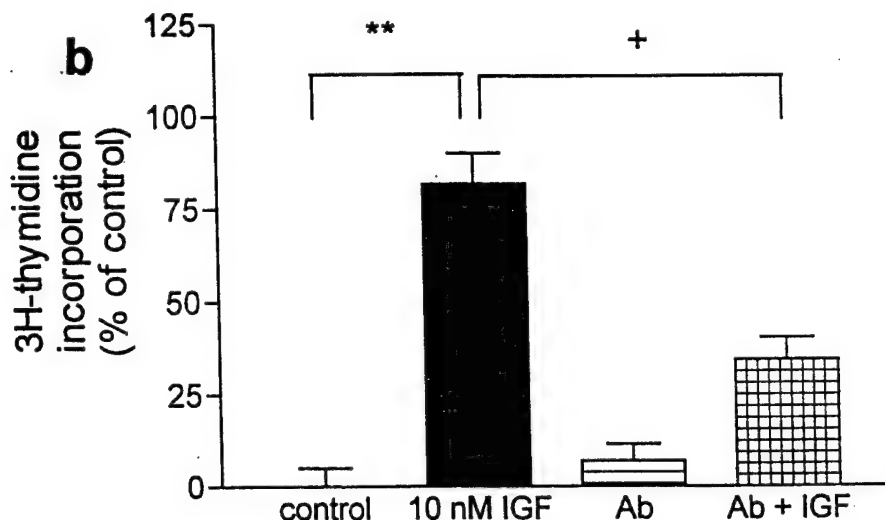


Figure 2b.  $\alpha$ IR3 (Ab) blocks the mitogenic effects of T3 and IGF-I. Quiescent MC3T3-E1 cells were incubated in 10 nM IGF-I or 1.5  $\mu$ g/ml  $\alpha$ IR3 alone for 24 hours or in the presence of both  $\alpha$ IR3 and T3. Control value: 2272  $\pm$  202 dpm/well; \* \* significantly different from control,  $p < 0.01$ . + significantly different from 10 nM T3,  $p < 0.05$ . 3H-thymidine incorporation is expressed as percent control. Data represent mean  $\pm$  sem,  $n = 3$  or 4 for each treatment group.

Effects of IGF-I receptor antagonists on T3 stimulated osteoblastic functions: 3H-Proline Incorporation - The effects of 72 hour T3 treatment on 3H-proline incorporation in confluent osteoblasts precultured in T3-free medium are illustrated in figure 3. 10 nM T3 caused a 41% increase in osteoblast 3H-proline incorporation. Under the conditions used, 10 nM IGF-I showed no stimulation. There was no synergism in the observed responses when the cells were treated with both T3 and IGF-I. (data not shown). When cells were treated with 10 nM T3 and in the presence of either  $\alpha$ IR3 or peptide analogue of IGF-I, JB1, the T3-stimulated 3H-proline incorporation was attenuated to the level of control. Although both  $\alpha$ IR3 and JB1 appear to have slight stimulatory effects on basal 3H-proline incorporation, these changes were not statistically significant.

Osteocalcin (OCN) - In a separate series of experiments, normal osteoblasts, grown to confluence and precultured in T3-free medium for 48 hours, were then incubated with either 1 or 10 nM T3 for 72 hours. The culture media were collected to assay for OCN production using RIA. Non-stimulated (control) osteoblasts produced an amount of OCN that was barely detectable with our assay. In media from control cells, OCN was 2.3  $\pm$  2.6 ng/ml (mean  $\pm$  sem,  $N=6$ ); and the lower resolution limit of our RIA was 1.56 ng/ml. As shown in figure 4a, 1 nM T3 induced approximately 50-fold increases OCN production by osteoblasts. There was a biphasic dose-dependent response to T3 in osteoblast OCN production with a peak at 1 nM; and, therefore, 1 nM T3 was used for OCN experiments. Similar to findings on 3H-proline



incorporation experiments, IGF-I (1 and 10 nM) did not stimulate detectable osteocalcin production under the conditions used. To determine whether T3-stimulated OCN production was mediated through an IGF-I pathway, cells were co-treated with 1 nM T3 and different concentrations of IGF-I receptor antibody,  $\alpha$ IR3. As shown in figure 4b, the  $\alpha$ IR3 antibody dose-dependently decreased T3-stimulated OCN production. Significant inhibition was observed ( $p < 0.01$ ) with 0.75  $\mu$ g/ml of antibody, and higher concentrations of antibody further inhibited T3-stimulated responses.

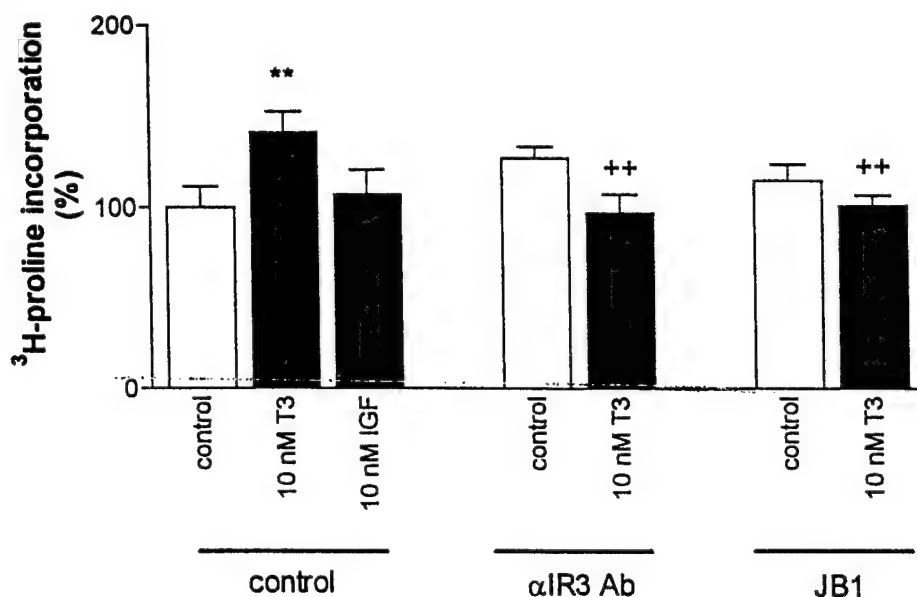


Figure 3. Antagonists to IGF-I receptor inhibit T3 stimulate 3H-proline incorporation in primary osteoblasts. Confluent osteoblasts were treated with 10 nM T3 for 72 hours alone or in the presence of either  $\alpha$ IR3 (Ab) or JB1. 3H-proline incorporation was determined and expressed as percent control. Control value: 23,806  $\pm$  2760 dpm/well; Data represent mean  $\pm$  sem,  $n = 4$  for each treatment group. \* \* significantly different from control,  $p < 0.01$ . ++ significantly different from 10 nM T3,  $p < 0.01$ .

**Alkaline phosphatase activity** - A stimulatory effect of T3 on MC3T3-E1 cells was evident when MC3T3-E1 cells grown for 18 days were treated with 10 nM T3 for 72 hours (total culture time of 21 days). T3-treated cells showed a 34% increase in ALP activity compared with non-treated cells (figure 6). 10 nM IGF-I treatment did not change ALP levels significantly. Co-treatment with 10 nM IGF and 10 nM T3 did not produce stimulation significantly different from treatment with 10 nM T3 alone (data not shown). The T3 stimulated ALP activity was attenuated in MC3T3-E1 transfected with an AS-ODN that is complimentary to mouse IGF-I receptor mRNA (figure 5). In these cells, T3 failed to elicit a significant increase in ALP. Cells transfected with 1.66  $\mu$ g/ml of the mismatch oligonucleotides did not cause an attenuated response to T3. Western immunoblotting for IGF-I receptor in MC3T3-E1 cell confirmed the specificity of AS-ODN for the IGF-I receptor. As shown in figure 6, MC3T3-E1 cells transfected with 1.66  $\mu$ g/ml AS-ODN showed a 40% decrease in IGF-I receptor expression. Cells transfected with same amount MS-ODN did not show decreased receptor expression. Transfection of the MC3T3-E1 cells with either AS-ODN or MS-ODN had no effect on the levels of actin protein expression.

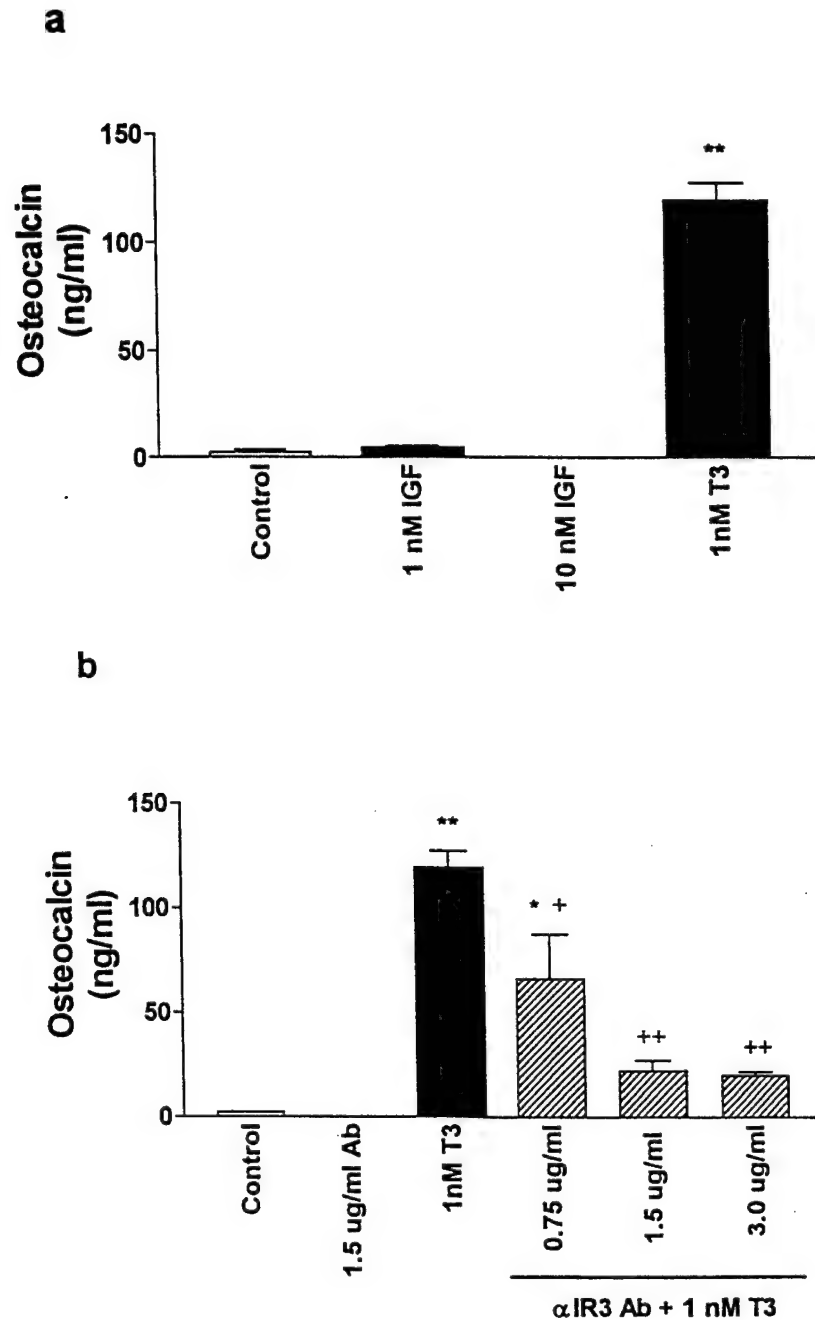


Figure 4.  $\alpha$ IR3 (Ab) blocks the effects of T3 on OCN. (a) Effects of T3 or IGF-I on primary osteoblast OCN production. Confluent osteoblasts were treated with either T3 or IGF-I for 72 hours. OCN was measured in the collected conditioned medium. \* \* significantly different from control,  $p < 0.01$ . (b) Confluent osteoblasts were incubated for 72 hours with 1 nM T3 alone or in the presence of different doses of  $\alpha$ IR3. Conditioned medium was collected and OCN measured by RIA. Data represent mean  $\pm$  sem,  $n = 3$  to 6 for each treatment group. \* significantly different from control,  $p < 0.05$ . ++ significantly different from 10 nM T3,  $p < 0.01$ .

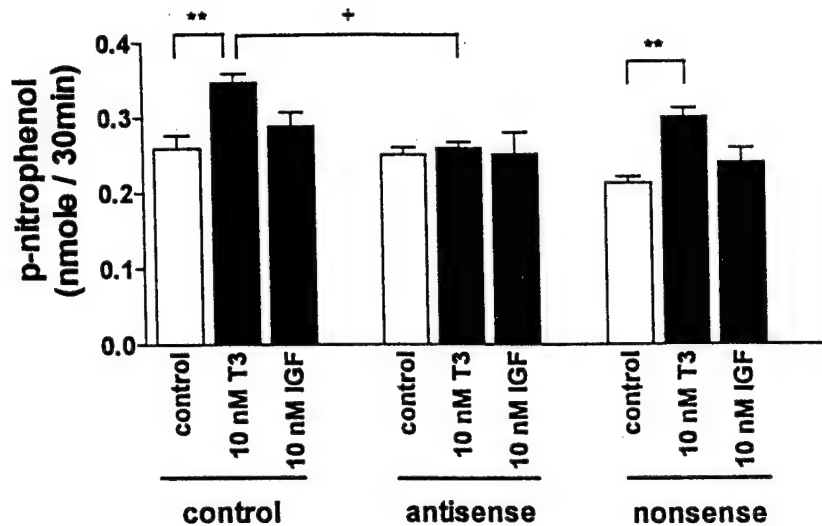


Figure 5. IGF-I receptor AS-ODN blocks T3 induced ALP activity in MC3T3-E1 cells. MC3T3-E1 cells were transfected with either AS-ODN or MS-ODN and treated with 10 nM T3 or 10 nM IGF-I for 72 hours. ALP level was assessed by the production of p-nitrophenol. Data represent mean  $\pm$  sem,  $n = 6$  to 12 for each treatment group. \* \* significantly different from the respective control,  $p < 0.01$ . + significantly different from non-transfected cells treated with 10 nM T3,  $p < 0.05$ .

#### Discussion (from manuscript - Specific Aim IB)

The importance of T3 to maintain normal skeletal physiology is well accepted. However, the mechanism of T3 effects on bone remodeling remains to be elucidated. We focused our project specifically on studying the anabolic actions of T3 on bone. These anabolic responses included osteoblast proliferation and osteoblast phenotypic markers such as ALP activity, OCN production, and collagen synthesis. An improved understanding of this basic anabolic process elicited by T3 could be beneficial in the management of skeletal problems in patients with altered thyroid status either due to disease processes or of iatrogenic origins, and is thus of clinical importance.

Our initial finding that T3 stimulates IGF-I production in UMR-106 cells and in fetal rat bone organ cultures (Lakatos et al. 1993), indicated the possibility that IGF-I may be responsible for some of the anabolic effects of T3 in bone tissues. The essential role of T3 on the skeleton can be seen from a case report of a patient with mutations of the T3 receptor gene showing the characteristic abnormal skeletal development (Refetoff et al. 1993). Hypothyroid patients, if untreated, often show a bone age delayed more than 2 standard deviations from their chronological age; and with subsequent thyroid replacement therapy, the bone age positively correlates with the concentration of serum T4 (Heyerdahl et al. 1994). Similarly, the importance of IGF-I to skeletal systems is evident from the delayed bone development in IGF-I gene knock-out mice; mice with IGF-I receptor mutations show significantly delayed or arrested ossification in the developing skeleton (Liu et al. 1993). These observations, taken together, further suggest the likelihood that IGF-I may be a candidate to mediate the stimulatory effects of T3 on bone formation. We concentrated our study on its effects on osteoblast functions.

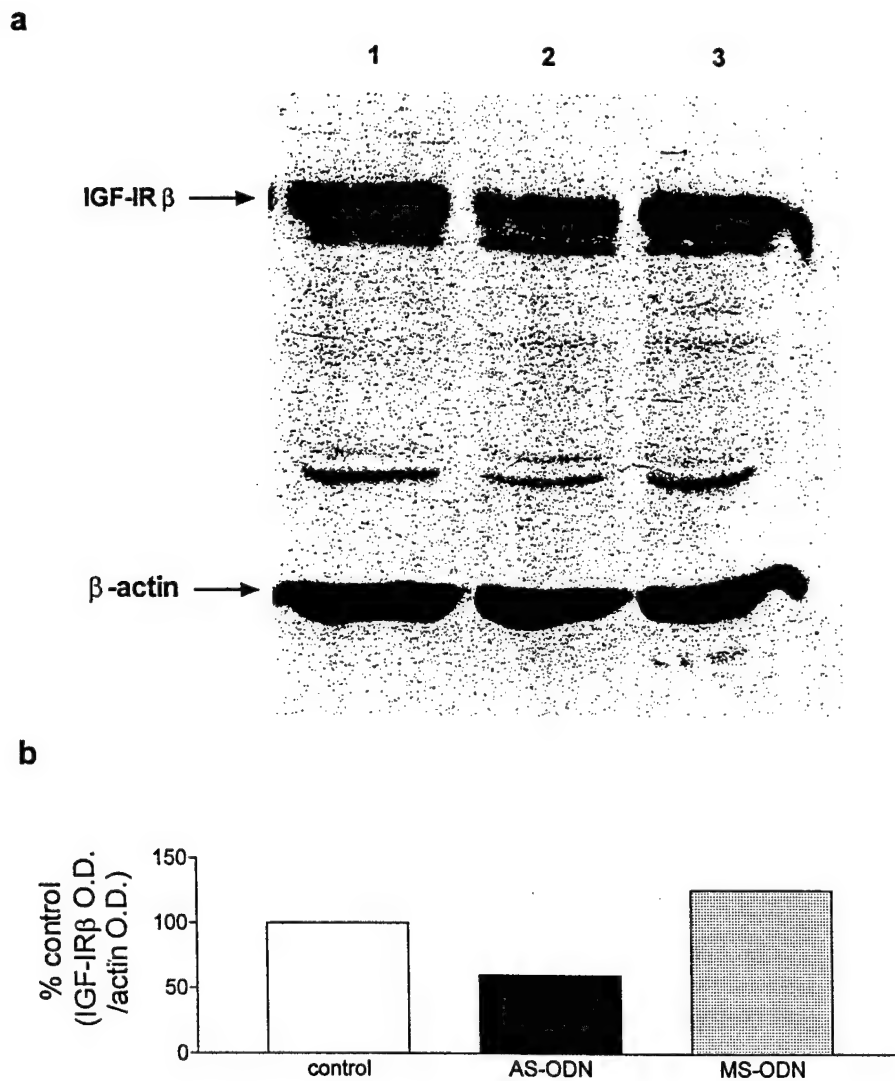


Figure 6. IGF-I receptor AS-ODN decreases IGF-I receptor expression in MC3T3-E1 cells. The IGF-I receptor protein level was determined by Western immunoblotting with an anti-IGF-I receptor beta antibody. (a) Levels of IGF-I receptor expression in control (lane 1) MC3T3-E1 cells and in cells transfected with either 1.66 ug/ml AS-ODN (lane 2) or MS-ODN (lane 3) were determined using western immunoblotting. Levels of beta-actin expression were measured and used for normalization. The experiments were repeated at least 3 times. (b) Expression was quantified by densitometry and expressed as the ratio of IGF-I receptor to actin. Data are presented as percent control.

Two different osteoblast models were used in this study to establish that the observed effects were not cell-line specific. Primary osteoblasts and MC3T3-E1 osteoblastic cells were used as model systems for our studies because these cells have the capacity to express phenotypic markers of osteoblast function and to synthesize IGF-I under basal and T3 stimulated conditions

(Stern 1996, Klaushofer et al. 1995). We determined the effects of T3 on ALP activity, OCN production, and 3H-proline incorporation because these parameters are generally accepted as osteoblast phenotypic markers. We chose to measure 3H-proline incorporation into total protein and not to discern between incorporation into collagenase-digestible protein and noncollagen protein because previous studies have shown that T3 stimulates 3H-proline incorporation into both collagen and noncollagen protein to a similar extent in bone (Kawaguchi et al. 1994). Assessment of ALP activity levels in different treatment conditions was limited to MC3T3-E1 cells because the treatment did not produce detectable changes in ALP levels in primary osteoblasts due to the high basal ALP activity in these cells. In addition, experiments using transfection of oligonucleotides were performed only in MC3T3-E1 cells because we were not successful in transfecting primary osteoblasts.

We employed different approaches to specifically interfere with the IGF-I actions in bone. Both the neutralizing antibody,  $\alpha$ IR3, and IGF-I peptide analogue, JB1, acted as antagonists to the IGF-I receptor to prevent binding of IGF-I. Both of these agents were shown to specifically block IGF-I stimulated 3H-thymidine incorporation in various cell-lines (Rohlik et al. 1987, Pietrzkowski et al. 1992). This effect was also seen in our cell models where we observed attenuated 3H-thymidine incorporation in response to IGF-I stimulation in cells co-treated with the antagonists. In a complementary approach, IGF-I action was reduced by decreasing IGF-I receptor number using antisense oligonucleotides specific for IGF-I receptor mRNA. The effectiveness of the AS-ODN was demonstrated using Western blotting for the IGF-I receptor. Using multiple approaches to interfere with IGF-I actions allowed us to eliminate the possibility that the observed responses were results of non-specific side-effects from methods used.

Our findings demonstrated anabolic effects of T3 on the osteoblast systems. T3 treatment caused a significant increase in phenotypic markers of osteoblasts. Primary osteoblasts treated with 10 nM T3 for 72 hr showed significant increases in both 3H-proline incorporation and osteocalcin production. To determine whether endogenously produced IGF-I was necessary for T3-stimulated osteoblastic responses, we tested the effect of a neutralizing anti-IGF-I receptor antibody and IGF-I peptide antagonist on T3 induced 3H-proline incorporation and osteocalcin production. Both the antibody and peptide antagonist had significant attenuating effects on T3-stimulated responses, indicating a requirement for IGF-I in T3-induced total protein and osteocalcin synthesis. Similar responses were also observed in alkaline phosphatase activity in MC3T3-E1 cells. Treatment with 10 nM T3 greatly stimulated alkaline phosphatase expression, and cells transfected with AS-ODN did not show increased alkaline phosphatase activity with the same T3 treatment. The MS-ODN, which we demonstrated by Western immunoblotting to have no effect on IGF-I receptor expression, did not have an attenuating effect on T3-stimulated alkaline phosphatase activity. This result further confirmed that the IGF-I pathway is necessary for T3-stimulated responses. We also attempted to reduce IGF-I receptor number in primary osteoblasts by transfecting them with oligonucleotides, however, we were not successful in transfecting these cells.

Our data also demonstrated mitogenic effects of T3. Most studies in the literature have shown that T3 has a dose-dependent, biphasic effect on osteoblast growth, with stimulation at low and inhibition at high concentrations (Allain and MacGregor 1993). At 10 nM T3 there was significant stimulation of 3H-thymidine incorporation in osteoblasts. 10 nM IGF-I also stimulated 3H-thymidine incorporation in osteoblasts. Consistent with our proposed hypothesis, treatment with neutralizing antibody to IGF-I receptor blocked both IGF-I and T3-stimulated thymidine

incorporation.

Given our results showing that agents which interfere with IGF-I actions can block T3-stimulated anabolic responses in osteoblasts, we predicted that treatment of IGF-I alone should increase the osteoblast phenotypic marker expression similar to that observed with T3 treated cells. Unexpectedly, in both models, treatment with IGF-I alone caused only a stimulation in mitogenic activity and did not increase the osteoblast phenotypic markers. Under the conditions used, treatment with various concentration of IGF-I alone did not produce a stimulation in 3H-proline incorporation, OCN production, or alkaline phosphatase expression and cells that were co-treated with both IGF-I and T3 did not produce responses that were significantly greater than cells treated with T3 alone. These data were surprising since previously published studies demonstrated IGF-I to have significant anabolic effects on bone (Canalis 1993, Centrella et al. 1990). IGF-I stimulated DNA synthesis and both collagen and non-collagen production in cultures of fetal rat calvaria and human osteoblasts (Canalis 1980, Wergehal et al. 1990). Pirskanen et al. showed that IGF-I plays an important stimulatory role modulating various steroid hormone effects on osteocalcin synthesis in MG-63 cells (Pirskanen et al. 1993). However, these studies were not performed in the same osteoblast systems and under the same conditions (e.g. resin-stripped serum) as our current study. It is possible that other factors could have been present that were removed by the stripping procedure, and these factors might complement IGF-I to stimulate anabolic actions in bone.

Furthermore, these differences in response to an IGF stimulus may reflect receptor population and receptor cross-reactivity and depend on cell type and osteoblast lineage (Conover 1996). This observation is also supported by recent studies that showed possible differential effects of thyroid hormones at different skeletal sites (Suwanwalaikorn et al 1996, Suwanwalaikorn et al. 1997). Since both of the cell models used in our study were calvarial derived, it would be of interest in future studies to determine whether the effects observed in the current study are also found in cells from other skeletal sites.

Interactions with various IGF binding proteins (IGFBPs) can also affect the observed responses. Both T3 and IGF-I can increase the production of IGFBPs (Schmid et al. 1992). IGFBPs can modulate IGF actions in bone. Certain IGFBPs inhibit IGF-I actions and others have been shown to have enhancing effects (Conover 1996, Feyen et al. 1991, Schmid et al. 1995). The relative amounts of the IGFBPs produced by T3 and IGF under our experimental conditions could play a role in the different responses seen since the final osteoblast response depends on presence or absence of endogenous IGFs and other IGFBPs.

We conclude from our findings that IGF-I is a necessary component for T3-stimulated anabolic osteoblast functions. It is unlikely that our findings are non-specific side effects of our interference approaches since different complementary methods of blocking IGF-I actions all attenuated T3-stimulated responses. The observation that IGF-I alone did not elicit the same responses as T3 suggests that IGF-I is a necessary but not sufficient factor and that other factors also contribute to the anabolic effects of T3 on bone.

Results from **Specific Aim IIB**, determining the role of IL-6 in the catabolic effects of T3 was described in the previous progress report, where we showed that antibodies to IL-6 and the IL-6 receptor attenuated the bone resorbing effects of T3. These results have been presented (Stern et al. 1998). However, the degree of inhibition was small, suggesting that this is only one



component of the mechanism of the effect of T3 to stimulate resorption. To focus more specifically on the potential role of IL-6 in T3 responses, during the past year we established in our laboratory the methodology for examining osteoclast formation in co-cultures of mouse marrow cells and normal neonatal mouse osteoblasts, a model in which IL-6 has been shown to promote osteoclastogenesis. Since we had no experience with this method, we took advantage of a 3 month visit by Dr. Je-Tae Woo, from the Tokyo Institute of Technology, who work focuses on effects of inhibitors on osteoclast precursor fusion and osteoclast formation. We have now established this model (see **Methods**) in our laboratory, and during the third year, we will carry out studies to a) confirm that IL-6 promotes osteoclastogenesis, b) establish whether T3 alone, or T3 plus IL-1 stimulate osteoclastogenesis in the model, and c) determine whether the antibodies to IL-6 and the IL-6 receptor prevent these effects. Also, we will carry out the proposed year 3 studies on interactions of T3 with other hormones on resorptive parameters, using both the fetal rat limb bone organ cultures and the osteoclastogenesis model.

## **Recommendations in Relation to the Statement of Work**

### **Specific Aims IA and IIA**

Difficulty in eliciting sufficiently large effects and consistent synergy between T3 and IL-1 on IL-6 expression in the osteosarcoma cell lines has led us to explore new models, specifically the use of the MC3T3-E1 cell line, which has yielded good T3 effects in our other studies (Specific Aim IB), and using neutral cell lines transfected with T3 receptors, transcription factors and IL-6 or IGF-I promoters in conjunction with second-messenger strategies to stimulate the IL-6 promoter. Based on recent reports of the involvement of estrogen receptors in thyroid receptor action on IL-6 and IGF-I genes, experiments during the third year will incorporate strategies for modifying the estrogen receptor in determining the pathway by which T3 affects IL-6 and IGF-I gene regulation.

### **Specific Aims IB and IIB**

Specific Aim IB has essentially been completed, with the demonstration, by three independent approaches, that interference with IGF-I action at the receptor level markedly attenuates the anabolic effects of T3. All of the proposed approaches were tested. The use of excess binding protein was not effective, and increasing the concentration of this to levels which might prove effect was prohibitively expensive. However, two approaches that were not originally proposed, an antibody to the IGF-I receptor, and a peptide antagonist to the IGF-I receptor were effective. Also, we tested several antisense constructs, and were successful in antagonizing the T3 responses by this approach as well. Specific Aim IIB is at the stage proposed for the end of the second year, with the demonstration, within the first year, of an inhibitory effect of antibodies to IL-6 and the IL-6 receptor to the resorptive effects of T3. However, this was not a complete inhibition, suggesting that the full response involves factors other than IL-6. To determine more precisely the role of IL-6, we will determine the effects of these antibodies on T3 and T3/IL-1 responses in an osteoclast differentiation model using marrow cultures. We will also examine the interactions between T3 and other hormones and cytokines on resorptive parameters, as proposed for Specific Aim IIB, in the third year.

## CONCLUSIONS

Our studies support the hypotheses that IGF-I and IL-6 are significant factors in the anabolic and catabolic effects, respectively of T3 on bone. The role of IGF-I is supported by the ability of antibody and antagonist peptide IGF-I receptor and antisense constructs to the IGF-I receptor to block the effects of T3. The evidence also shows that IGF-I is critical to the T3 effect, but possibly not a sufficient factor, since it did not by itself elicit any of the anabolic effects other than cell proliferation. Other factors, in addition to IGF-I, present in the culture system must be contributing to the anabolic effects of T3. It is interesting to note that in our initial findings that led to this proposal we found that higher, supraphysiologic concentrations of T3 resulted in lesser stimulation of IGF-I production. This loss of the IGF-I response could be one mechanism by which hyperthyroidism leads to bone loss. Another mechanism is the proposed involvement of IL-6, a cytokine that leads to osteogenesis. Evidence supporting this are our findings that antibodies to IL-6 and the IL-6 receptor can attenuate the resorptive responses to T3. Studies proposed for the third year would look more specifically for IL-6 involvement in T3 responses in an osteoclastogenesis model based upon marrow precursor cell differentiation.

The studies of the mechanisms by which T3 stimulates IGF-I and IL-1 production have been more problematic. A range of cell lines human, and rodent have been tested, and the findings have not been consistent enough or of sufficient magnitude to permit the types of molecular mechanism studies proposed. We are now exploring the MC3T3-E1 murine cell line that we have used for other studies. We had previously not chosen to use this cell line because of the time required for it to differentiate. However, this closer similarity to normal osteoblastic cells may prove to be an advantage, and we are now focussing on this line. Also, we are trying to utilize neutral cells transfected with the necessary components of the pathway as a cleaner model to dissect out the steps, and also to take advantage of recent findings regarding thyroid hormone and estrogen receptors in mediating responses to T3.

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